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(54) Title: PHARMACEUTICAL COMPOSITION FOR IMMUNOMODULATION AND PREPARATION OF VACCINES COM-  
PRISING AN ANTIGEN AND AN IMMUNOGENIC OLIGODEOXYNUCLEOTIDE AND A POLYCATIONIC POLYMER AS  
ADJUVANTS

(57) Abstract: The invention discloses a pharmaceutical composition comprising an antigen, an immunogenic CpG-ODN and a  
polycationic polymer.

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PHARMACEUTICAL COMPOSITION FOR IMMUNOMODULATION AND PREPARATION OF VACCINES COMPRISING AN ANTIGEN AND AN IMMUNOGENIC OLIGODEOXYNUCLEOTIDE AND A POLYCATIONIC POLYMER AS ADJUVANTS

The invention relates to a pharmaceutical composition, e.g. to be used for immunomodulation especially as vaccines.

Vaccines can save more lives (and resources) than any other medical intervention (Nossal, 1998). Owing to world-wide vaccination programs the incidence of many fatal diseases has been decreased drastically. Although this notion is valid for a whole panel of diseases, e.g. tuberculosis, diphtheria, pertussis, measles and tetanus, there are no effective vaccines for numerous infectious disease including most viral infections, such as AIDS. There are also no effective vaccines for other diseases, infectious or non-infectious claiming millions the lifes of millions of patients per year including malaria or cancer. In addition, the rapid emergence of antibiotic-resistant bacteria and microorganisms calls for alternative treatments with vaccines being a logical choice. Finally, the great need for vaccines is also illustrated by the fact that infectious diseases, rather than cardiovascular disorders or cancer or injuries remain the largest cause of death and disability in the world (Bloom and Widdus, 1998).

From an immunological point of view one major problem in the field of vaccines today is that traditional vaccines (and/or the immune-modulating compounds contained within these preparations) are designed to induce high levels of antibodies (Harlow and Lane, 1988). However, antibodies on their own are not effective in preventing a large number of diseases including most illnesses caused by viruses, intracellular bacteria, certain parasites and cancer. Examples for such diseases are, but are not restricted to, the above-mentioned HIV virus or Plasmodium spec. in case of malaria. In numerous experimental systems it has been shown that the cellular arm of the immune system, including T cells, rather than the humoral arm, is important for these indications. Therefore, novel, innovative technologies are needed to overcome the limitations of conventional vaccines. The focus must be on technologies that reliably induce the cellular immune system, including antigen specific T cells, which recognize molecules expressed on pathogen infected cells. Ideally, vaccines are designed that

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induce both T cells distinguishing diseased and/or infected cells from normal cells and, simultaneously, antibodies secreted by B cells recognizing pathogens in extracellular compartments.

Several established vaccines consist of live attenuated organism where the risk of reversion to the virulent wild-type strain exists. In particular in immunocompromised hosts this can be a live threatening scenario. Alternatively, vaccines are administered as a combination of pathogen-derived antigens together with compounds that induce or enhance immune responses against these antigens (these compounds are commonly termed adjuvant), since these subunit vaccines on their own are generally not effective.

Whilst there is no doubt that the above vaccines are valuable medical treatments, there is the disadvantage that, due to their complexity, severe side effects can be evoked, e.g. to antigens that are contained in the vaccine that display crossreactivity with molecules expressed by cells of vaccinated individuals. In addition, existing requirements from regulatory authorities, e.g. the World Health Organization (WHO), the Food and Drug Administration (FDA), and their European counterparts, for exact specification of vaccine composition and mechanisms of induction of immunity, are difficult to meet.

Some widely used vaccines are classified in table 1.

Table 1

Category of vaccine	Example
<b>Whole cell</b>	
Attenuated bacteria or viruses	Bacille Calmette-Guerin (BCG) (tuberculosis) Measles Mumps Rubella Oral Polio Vaccine (Sabin)
Killed bacteria or viruses	Pertussis Inactivated polio vaccine (Salk)
<b>Subunit</b>	
Toxoid	Diphtheria Tetanus
Capsular polysaccharide	H. influenzae type B
Yeast recombinant subunit	Hepatitis B surface protein

From (Liljeqvist and Stahl, 1999) with modifications.

Antigen presenting cells belong to the innate immune system, which has evolved as a first line host defense that limits infection early after exposure to microorganisms (Hoffmann et al., 1999). Cells of the innate immune system recognize patterns or relatively non-specific structures expressed on their targets rather than more sophisticated, specific structures which are recognized by the adaptive immune system (Hoffmann et al., 1999). Examples of cells of the innate immune system are macrophages and dendritic cells but also granulocytes (e.g. neutrophils), natural killer cells and others. By contrast, cells of the adaptive immune system recognize specific, antigenic structures, including peptides, in the case of T cells and peptides as well as three-dimensional structures in the case of B cells. The adaptive immune system is much more specific and sophisticated than the innate immune system and improves upon repeat exposure to a given pathogen/antigen. Phylogenetically, the innate immune system is much older and can be found already in very primitive organisms. Nevertheless, the innate immune system is critical during the

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initial phase of antigenic exposure since, in addition to containing pathogens, cells of the innate immune system, i.e. APCs, prime cells of the adaptive immune system and thus trigger specific immune responses leading to clearance of the intruders. In sum, cells of the innate immune system and in particular APCs play a critical role during the induction phase of immune responses by a) containing infections by means of a primitive pattern recognition system and b) priming cells of the adaptive immune system leading to specific immune responses and memory resulting in clearance of intruding pathogens or of other targets (Roitt et al., 1998). These mechanisms may also be important to clear or contain tumor cells.

As mentioned above, cells of the innate immune system recognize patterns expressed on their respective targets. Examples are lipopolysaccharides (LPS) in the case of Gram-negative bacteria, mycobacterial glycolipids, lipoteichoic acids of Gram-positive bacteria, mannans of yeast and double stranded RNAs of viruses (Hoffmann et al., 1999). In addition they may recognize patterns such as altered glycosylations of proteins on tumor cells.

Recent findings describe DNAs of protozoae or lower eukaryotes as a further pattern recognized by the innate (but possibly also by the adaptive) immune system of mammals (and probably most if not all vertebrates) (Krieg, 1996; Lipford et al., 1998).

The immune system recognizes DNA of lower organisms including bacteria probably due to structural and sequence usage differences between pathogen and host DNA. In particular short stretches of DNA, derived from non-vertebrates or in form of short oligodeoxynucleotides (ODNs) containing nonmethylated cytosine-guanine dinucleotides (CpG) in a certain base context, are targeted (Krieg et al., 1995). CpG motifs are found at the expected frequency in bacterial DNA but are much less frequent in vertebrate DNA (Lipford et al., 1998; Pisetsky, 1999). In addition, non-vertebrate (i.e. bacterial) CpG motifs are not methylated whereas vertebrate CpG sequences are. These differences between bacterial DNA and vertebrate DNA allow vertebrates to recognize non-vertebrate DNA.

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Natural CpG-containing DNA, ODNs, as well as thiophosphate-substituted (exchange of thiophosphate residues for phosphate) ODNs containing CpG motifs (CpG-ODN) are not only potent activators of immune cell proliferation and humoral immune responses (Krieg et al., 1995), but also stimulate strong cellular immune responses (reviewed in (Lipford et al., 1998)). DNA/ODNs containing non-methylated CpG motifs can directly activate monocytic cells (dendritic cells, macrophages) and B cells. Likely, natural killer (NK) cells are not directly activated but respond to monocyte-derived IL-12 (interleukin 12) with a marked increase in their IFN- $\gamma$  production (Chace et al., 1997). In consequence, the induction of monocytes and NK cells by CpG DNA promotes the induction of Th1-type responses and the development of cytotoxic T cells.

Dendritic cells, which represent important antigen presenting cells during primary immune responses, mature in vitro under the influence of CpG-ODN (Hartmann et al., 1999; Sparwasser et al., 1998) and produce large amounts of IL-12, TNF- $\alpha$ , IL-6 and to a lesser extent IL-10 (Klinman et al., 1996; Sparwasser et al., 1998) when exposed to these compounds. Thus, one characteristic effect of bacterial DNA and CpG-ODNs is the induction of Th1 type humoral and cell-mediated responses to protein antigens, which is characterized by it's specific cytokine pattern (Mosmann et al., 1986). ODNs containing CpG motifs have been used as a vaccine adjuvant in mice to enhance for example the antibody response to a tetanus vaccine (Krieg et al., 1998) or to promote a strong antigen-specific Th1 cytokine response in an experimental model of virus infection (Oxenius et al., 1999). CpG DNA is also described as a strong adjuvant for antibody and cytotoxic T lymphocyte (CTL) responses against Hepatitis B virus antigens (Davis et al., 1998). The induction of strong Th1 cytokine and CTL responses indicates that CpG-ODN may also be useful for cancer vaccines using tumor antigens. First published data show that mice immunized with the idiotype from a B lymphoma in combination with a CpG-ODN as an adjuvant displayed prolonged survival (Weiner et al., 1997).

Although CpG ODNs or non-methylated DNA containing CpG motifs are potentially very powerful adjuvants, there is a more sinister

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side to this technology (Pisetsky, 1997). For example, it has been reported that with high doses of bacterial DNA containing non-methylated CpG motifs septic shock can be induced (Sparwasser et al., 1997) under certain circumstances. This is likely due to excessive amounts of cytokines, in particular TNF- $\alpha$  and IL-6, but also others, that are secreted by cells upon exposure to CpG-ODN or to bacterial DNA (Sparwasser et al., 1997). Bacterial DNA and ODN may also be the cause of inflammatory processes which are a common complication in lung infections (Schwartz et al., 1997). Furthermore, it is suspected that early gene therapy trials, where formulated or non-formulated plasmid DNA-containing non-methylated CpG motifs was administered to the lung of cystic fibrosis patients failed, because of strong inflammatory processes, which were shown to be caused by CpG motifs (Paillard, 1999; Yew et al., 1999). CpG sequences contained in plasmid DNA also appear to be responsible for deaths of animals following intravenous injections of plasmid DNA formulated with liposomes (Paillard, 1999). Finally, animals exposed to high concentrations of CpG ODN develop symptoms of arthritis, likely due to inflammatory processes caused by the ODN (Deng et al., 1999).

Collectively, these reports highlight drawbacks of ODN adjuvants, which may be circumvented if the amount of ODNs to be used for a vaccine can be lowered significantly.

Polycationic polymers, for example the polycationic amino acid polymers poly-L-arginine and poly-L-lysine, have been shown to allow very efficient charging of antigen presenting cells (APCs) with antigens in vitro and in vivo (Buschle et al., Gene Ther Mol Biol 1, (1998), 309-321; Buschle et al., Proc Natl Acad Sci USA 94, (1997), 3256-3261; Schmidt et al., Proc Natl Acad Sci USA 94, (1997), 3262-3267). This is thought to be the key event for triggering immune cascades, eventually leading to the induction of antigen specific immune effector cells that are able to destroy or neutralize targets. It has been shown previously that a number of polycationic compounds exert effects on immune cells (Buschle et al., Gene Ther Mol Biol 1, (1998), 309-321; Buschle et al., Proc Natl Acad Sci USA 94, (1997), 3256-3261).

Co-injection of a mixture of poly-L-arginine or poly-L-lysine to-

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gether with an appropriate antigen as a vaccine protect animals from tumor growth in several animal models (Buschle et al., Gene Ther Mol Biol 1, (1998), 309-321; Schmidt et al., Proc Natl Acad Sci USA 94, (1997), 3262-3267). Thus, a vaccine consisting of polycationic compounds and antigen(s) is accepted in the art as being a very effective form of treatment.

It is the object of the present invention to provide a pharmaceutical composition which allows an effective delivery to a target cell especially to the cellular immune system, but also to other cell types in order to induce potent immune responses. It is a further object of the invention to provide means to decrease or even ablate undesired immune responses.

This objective is solved by a pharmaceutical composition comprising

- an antigen or an immunosuppressing agent
- an immunogenic ODN, containing CpG motifs, and
- a polycationic polymer.

Surprisingly, it has turned out that the combination of the immunogenic ODN which may also include chemotactic or differentiation inducing factor as immunostimulating substance and the polycationic polymer with an antigen according to the present invention leads to a synergistic immunomodulating effect for a given antigen preparation. The combination of antigen, immunogenic ODN and polycationic polymer allowed the generation of superior vaccines as compared to vaccines consisting of immunogenic ODN and antigen or antigen and polycationic polymer. In fact, this was observed even with suboptimal amounts of the immunogenic ODNs. Although such ODNs as well as the polycationic polymers have been known in the art (as well as many other substances for which an adjuvant effect is reported) as being potent factors in antigen preparations, the combination of these substances shows an effect which is by far better than only the combination of the single isolated effects of the compounds. In contrast to other combinations of different classes of adjuvants, where - at least - only additive effects are observed (if it all), the selection of the polycationic polymers and the immunogenic ODNs in a combined application



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together with an antigen showed an unforeseeable increase of effectiveness in immune response to a selected antigen. In a synergistic manner, the immunogenic ODNs and the polycationic polymers allow a very efficient cellular immune response as well as an immunomodulating effect which enables a superior vaccination perspective.

The administration of ODNs with CpG motifs also resulted in the induction of adverse side reactions, especially in the systemic release of pro-inflammatory cytokines, like TNF- $\alpha$  and IL-6. It was very surprising that these side effects can be prevented by providing a polycationic polymer together with the ODN and the antigen. Especially polycationic polymers comprising peptide bonds show an almost complete prevention of TNF- $\alpha$  and IL-6.

The antigens to be used in the present compositions are not critical. A vaccine can contain a whole variety of different antigens. Examples of antigens are whole-killed organisms such as inactivated viruses or bacteria, fungi, protozoa or even cancer cells. Antigens may also consist of subfractions of these organisms/tissues, of proteins, or, in their most simple form, of peptides. Antigens can also be recognized by the immune system in form of glycosylated proteins or peptides and may also be or contain polysaccharides or lipids. Short peptides can be used since for example cytotoxic T cells (CTL) recognize antigens in form of short usually 8-11 amino acids long peptides in conjunction with major histocompatibility complex (MHC) (Rammensee et al., Immunogenetics 41, (1995), 178-228). B cells recognize longer peptides starting at around 15 amino acids (Harlow et al, Cold Spring Harbor: Cold Spring Harbor Laboratory, (1988)). By contrast to T cell epitopes, the three dimensional structure of B cell antigens may also be important for recognition by antibodies. In order to obtain sustained, antigen-specific immune responses, adjuvants may help to trigger immune cascades that involve all cells of the immune system necessary. Primarily, adjuvants are acting, but are not restricted in their mode of action, on so-called antigen presenting cells (APCs). These cells usually first encounter the antigen(s) followed by presentation of processed or unmodified antigen to immune effector cells. Intermediate cell types may also be involved. Only effector cells with the appropriate speci-

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ficity are activated in a productive immune response. The adjuvant may also locally retain antigens and co-injected other factors. In addition the adjuvant may act as a chemoattractant for other immune cells or may act locally and/or systemically as a stimulating agent for the immune system.

Preferably proteins or peptides derived from a viral or a bacterial pathogen or from fungi or parasites are used as such antigens (including derivatized antigens or glycosylated or lipidated antigens or polysaccharides or lipids). Another preferred source of antigens are tumor antigens. Preferred pathogens are selected from human immunodeficiency virus (HIV), hepatitis A and B viruses, hepatitis C virus (HCV), rous sarcoma virus (RSV), Epstein Barr virus (EBV) Influenza virus, Rotavirus, Staphylococcus aureus, Chlamydia pneumoniae, Chlamydia trachomatis, Mycobacterium tuberculosis, Streptococcus pneumoniae, Bacillus anthracis, Vibrio cholerae, Plasmodium sp. (Pl. falciparum, Pl. vivax, etc.), Aspergillus sp. or Candida albicans. Antigens may also be molecules expressed by cancer cells (tumor antigens). The derivation process may include the purification of a specific protein from the pathogen/cancer cells, the inactivation of the pathogen as well as the proteolytic or chemical derivatization or stabilization of such a protein. In the same way also tumor antigens (cancer vaccines) or autoimmune antigens may be used in the pharmaceutical composition according to the present invention. With such compositions a tumor vaccination or a treatment for autoimmune diseases may be performed.

In the case of peptide antigens the use of peptide mimitopes/agonists/superagonists/antagonists or peptides changed in certain positions without affecting the immunologic properties or non-peptide mimitopes/agonists/superagonists/antagonists (reviewed in Sparbier and Walden, 1999) is included in the current invention. Peptide antigens may also contain elongations either at the carboxy or at the amino terminus of the peptide antigen facilitating interaction with the polycationic compound(s) or the immunostimulatory compound(s). For the treatment of autoimmune diseases peptide antagonists may be applied.

Antigens may also be derivatized to include molecules enhancing

antigen presentation and targeting of antigens to antigen presenting cells.

In one embodiment of the invention the pharmaceutical composition serves to confer tolerance to proteins or protein fragments and peptides which are involved in autoimmune diseases. Antigens used in this embodiments serve to tolerize the immune system or down-regulate immune responses against epitopes involved in autoimmune processes.

The immunogenic ODN according to the present invention can be of prokaryotic and eukaryotic origin. In the case of eukaryotic origin, DNA should be derived from, based on the phylogenetic tree, less developed species (e.g. insects, but also others). In a preferred embodiment of the invention the immunogenic ODN is a synthetically produced DNA-molecule or mixtures of such molecules. Derivates or modifications of ODNs such as thiophosphate substituted analogues (thiophosphate residues substitute for phosphate) as for example described in US patents US 5,723,335 and US 5,663,153, and other derivatives and modifications, which preferably stabilize the immunostimulatory composition(s) but do not change their immunological properties (e.g. Sparbier and Walden, 1999), are also included. A preferred sequence motif is a six base DNA motif containing an (unmethylated) CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (5'-Pur-Pur-C-G-Pyr-Pyr-3') (Pisetsky, 1999). The CpG motifs contained in the ODNs according to the present invention are more common in microbial than higher vertebrate DNA and display differences in the pattern of methylation. (Bird, A.P., Nature 1986, 321:209; Pisetsky, D.S., Immunol Res 1999, 19 (1): 35-46). (Lipford et al., 1998). Surprisingly, sequences stimulating mouse APCs are not very efficient for human cells (Hartmann et al., 1999; Krieg, 1999). Preferred ODNs according to the present invention are disclosed e.g. in EP 0 468 520 A2, WO 96/02555, WO 98/16247, WO 98/18810, WO 98/37919, WO 98/40100, WO 98/52581, WO 98/52962, WO 99/51259 and WO 99/56755 all incorporated herein by reference. Apart from stimulating the immune system certain ODNs are neutralizing some immune responses (Krieg, 1999; Lipford et al., 1998). These sequences are also included in the current invention, for example for applications for the treatment of autoim-

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mune diseases. ODNs/DNAs may be produced chemically or recombinantly or may be derived from natural sources. Preferred natural sources are insects. Of course, also mixtures of different ODNs may be used according to the present invention.

The polycationic compound(s) to be used according to the present invention may be any polycationic compound which shows the characteristic effect according to the WO 97/30721. Preferred polycationic compounds are selected from basic polypeptides, organic polycations, basic polyaminoacids or mixtures thereof. These polyaminoacids should have a chain length of at least 4 amino acid residues (see: Tuftsin as described in Goldman et al (1983)). Especially preferred are substances containing peptidic bounds, like polylysine, polyarginine and polypeptides containing more than 20%, especially more than 50% of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof. Other preferred polycations and their pharmaceutical compositions are described in WO 97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably these polypeptides contain between 20 and 500 amino acid residues, especially between 30 and 200 residues.

These polycationic compounds may be produced chemically or recombinantly or may be derived from natural sources.

Cationic (poly)peptides may also be polycationic anti-bacterial microbial peptides with properties as reviewed in (Ganz and Lehrer, 1999; Hancock, 1999). These (poly)peptides may be of prokaryotic or animal or plant origin or may be produced chemically or recombinantly (Andreu and Rivas, 1998; Ganz and Lehrer, 1999; Simmaco et al., 1998). Peptides may also belong to the class of defensins (Ganz, 1999; Ganz and Lehrer, 1999). Sequences of such peptides can be, for example, be found in the Antimicrobial Sequences Database under the following internet address:

<http://www.bbcm.univ.trieste.it/~tossi/pag1.html>

Such host defense peptides or defensives are also a preferred form of the polycationic polymer according to the present invention. Generally, a compound allowing as an end product activation (or down-regulation) of the adaptive immune system, preferably

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mediated by APCs (including dendritic cells) is used as polycationic polymer.

Especially preferred for use as polycationic substance in the present invention are cathelicidin derived antimicrobial peptides or derivatives thereof (A 1416/2000, incorporated herein by reference), especially antimicrobial peptides derived from mammal cathelicidin, preferably from human, bovine or mouse.

Polycationic compounds derived from natural sources include HIV-REV or HIV-TAT (derived cationic peptides, antennapedia peptides, chitosan or other derivatives of chitin) or other peptides derived from these peptides or proteins by biochemical or recombinant production. Other preferred polycationic compounds are cathelin or related or derived substances from cathelin. For example, mouse cathelin is a peptide which has the amino acid sequence  $\text{NH}_2\text{-RLAGLLRKGGKIGEKLLKKIGOKIKNFFQKLVPQPE-COOH}$ . Related or derived cathelin substances contain the whole or parts of the cathelin sequence with at least 15-20 amino acid residues. Derivations may include the substitution or modification of the natural amino acids by amino acids which are not among the 20 standard amino acids. Moreover, further cationic residues may be introduced into such cathelin molecules. These cathelin molecules are preferred to be combined with the antigen and the immunogenic ODN according to the present invention. However, these cathelin molecules surprisingly have turned out to be also effective as an adjuvant for a antigen without the addition of further adjuvants. It is therefore possible to use such cathelin molecules as efficient adjuvants in vaccine formulations with or without further immunactivating substances.

Another preferred polycationic substance to be used according to the present invention is a synthetic peptide containing at least 2 KKK-motifs separated by a linker of 3 to 7 hydrophobic amino acids (A 1789/2000, incorporated herein by reference).

It was very surprising that with the pharmaceutical composition according to the present invention the immunostimulating effect was significantly higher than it could be expected from the addition of the effects of each single component or even the addition

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of the effects of the polycation with the antigen and the immunogenic ODN with the antigen. Moreover, it turned out that the effect of the immunogenic ODN alone is not very high when an antigen is directly applied with this substance. This is true in particular if the compounds are not repeatedly administered. Very importantly combination of the compounds allows to use less of the immunogenic ODN which may help to avoid side effects (see above). Surprisingly, also side effects being present when ODNs are administered are prevented or reduced by the combined administration of antigen, ODN and polycationic polymer.

According to another aspect the present invention also relates to vaccines which comprise a composition according to the present invention.

Moreover, the present invention is also drawn to the use of the composition according to the present invention for manufacturing a vaccine.

The relative amounts of the ingredients of the present composition are highly depending on the necessities of the individual composition, e.g. the polycationic polymer to be used. In the case of poly-L-arginine and poly-L-lysine, preferred amounts of antigen/immunogenic ODN/immunosuppressive compound polycation lie in the range of 1-10000 µg antigen per vaccination, 1pM - 1mM immunogenic ODN per dose, and 0,1 to 1000 µg polycation per vaccination.

It is especially preferred to provide CpG ODNs and polycationic compounds (especially polycationic polypeptides, such as poly-arginine or poly-lysine) in a charge ratio of between 100:1 and 0,1:1, preferably between 50:1 and 0,5:1, especially between 10:1 and 0,5:1.

The present compositions may be applied to a patient, e.g. a vaccination candidate, in efficient amounts e.g. by weekly, bi-weekly or monthly intervals. Patients to be treated with the present compositions may also be vaccinated repeatedly or only once. A preferred use of the present invention is the active immunisation, especially of humans or animals without protection against

the specific antigen.

The route of application for the present composition is not critical, e.g. subcutaneous, intramuscular, intradermal or transdermal injection is suitable as well as oral uptake. The adaption of the present composition to such an application route is easily conducted by the man skilled in the art.

It is also possible to apply the present composition separately e.g. by injecting the immunogenic ODN separately from the antigen/polycation composition. The present invention is therefore also directed to a kit comprising a composition containing the antigen and the polycationic polymer as one component and a composition containing the immunogenic ODN substance as a second component.

The components may be applied at the same site or time, however, an application at different sites or at a different time or for a different time period is also possible. It is also possible to vary the systemic or local applications of the composition or the components, respectively.

Another aspect of the present invention relates to a kit comprising a component containing an immunogenic ODN, a component containing a polycationic polymer and a component containing an antigen. Preferably the antigen is provided already mixed with the polycationic polymer.

The invention will be described in more detail by way of the following examples and the drawing figures, yet it is not restricted to these particular embodiments.

Fig. 1A and 1B: the IFN- $\gamma$ -ELISPOT of the immune response against the OVA-peptide SIINFEKL;

Fig. 2: the IFN- $\gamma$ -ELISPOT of the immune response against the mouse mastocytoma P815-derived peptide PlA (Fig. 2A), the CSP-peptide SYVPSAEQI (Fig. 2B), the LLO peptide GYKDGNEYI (Fig. 2C) and the OVA peptide ISQAVHAAHAEINE (Fig. 2D);

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Fig. 3: the IFN- $\gamma$ -ELISPOT of the immune response against the MC1R peptide WGPFFLHL;

Fig. 4: the IFN- $\gamma$ -ELISPOT of the immune response at different injection sites against the OVA-peptide SIINFEKL; and

Fig. 5: the combined application of CpG-ODNs and poly-L-arginine (pR 60) prevents the induction of systemic TNF- $\alpha$  and IL-6 production.

### EXAMPLES

In all experiments thiophosphate-substituted ODNs (with thiophosphate residues substituting for phosphate, hereafter called "thiophosphate substituted oligodeoxynucleotides") were used since such ODNs display higher nuclease resistance (Ballas et al., 1996; Krieg et al., 1995; Parronchi et al., 1999).

Lymph nodes were passed through a 70  $\mu$ m cell strainer and washed twice with DMEM medium (GIBCO BRL) containing 5% fetal calf serum (FCS, SIGMA chemicals). Cells were adjusted to  $10^7$  cells/ml in DMEM/5%FCS. IFN- $\gamma$ -ELISPOT assay were carried out in duplicates as described (Miyahira et al., 1995). This method is a widely used procedure allowing the quantification of antigen-specific T cells. Lymphocytes were stimulated ex vivo in duplicates with medium (background), pR 60, CpG-ODN and Concanavalin A (Con A). Spots representing single IFN- $\gamma$  producing T cells were counted and the number of background spots was subtracted from all samples. There were many spots detected after the stimulation with Con A (data not shown) indicating a good condition of the used lymphocytes.

**Example 1 :** The combined application of CpG-ODN and poly-L-arginine (pR 60) strongly enhances the induction of Ovalbumin-peptide specific T cells in a concentration (pR 60)-dependent manner.

Mice	C57Bl/6 (Harlan/Olac)
Peptide	OVA <sub>257-264</sub> -Peptide (SIINFEKL), a MHC class I (H-2Kb)-



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restricted epitope of chicken ovalbumin (Rotzschke et al., 1991), was synthesized using standard solid phase F-moc synthesis, HPLC purified and analysed by mass spectroscopy for purity.

Dose: 300 µg/mouse

**Poly-L-Arginine**

ne 60 (pR60) Poly-L-Arginine with an average degree of polymerization of 60 arginine residues; SIGMA chemicals  
Dose: 1000, 100 or 10 µg/mouse

CpG-ODN 1668 thiophosphate substituted ODNs containing a CpG motif: TCC ATG ACG TTC CTG ATG CT, synthesized by NAPS GmbH, Göttingen.

Dose: 5 nmol/mouse

**Non-CpG-ODN**

1911 phosphothioates modified oligodinucleotides containing no CpG motif: TCC AGG ACT TTC CTC AGG TT, synthesized by NAPS GmbH, Göttingen.

Dose: 5 nmol/mouse

Experimental groups (5 mice per group)

1. OVA<sub>257-264</sub>-Peptide + CpG-ODN
2. OVA<sub>257-264</sub>-Peptide + CpG-ODN + pR 60 1000 µg
3. OVA<sub>257-264</sub>-Peptide + CpG-ODN + pR 60 100 µg
4. OVA<sub>257-264</sub>-Peptide + CpG-ODN + pR 60 10 µg
5. OVA<sub>257-264</sub>-Peptide + Non-CpG-ODN
6. OVA<sub>257-264</sub>-Peptide + Non-CpG-ODN + pR 60 1000 µg
7. OVA<sub>257-264</sub>-Peptide + Non-CpG-ODN + pR 60 100 µg
8. OVA<sub>257-264</sub>-Peptide + Non-CpG-ODN + pR 60 10 µg
9. OVA<sub>257-264</sub>-Peptide + pR 60 1000 µg
10. OVA<sub>257-264</sub>-Peptide + pR 60 100 µg
11. OVA<sub>257-264</sub>-Peptide + pR 60 10 µg

On day 0 mice were injected into each hind footpad with a total volume of 100 µl (50 µl per footpad) containing the above mentioned compounds. Animals were sacrificed 4 days after injection and popliteal lymph nodes were harvested. Lymph nodes were passed through a 70 µm cell strainer and washed twice with DMEM medium (GIBCO BRL) containing 5% fetal calf serum (FCS, SIGMA chemicals). Cells were adjusted to 10<sup>7</sup> cells/ml in DMEM/5%FCS.

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IFN- $\gamma$ -ELISPOT assay were carried out in duplicates as described (Miyahira et al., 1995). This method is a widely used procedure allowing the quantification of antigen-specific T cells. Lymphocytes were stimulated ex vivo in duplicates with medium (background), pR 60, CpG-ODN and Concanavalin A (Con A). Spots representing single IFN- $\gamma$  producing T cells were counted and the number of background spots was subtracted from all samples. There were many spots detected after the stimulation with Con A (data not shown) indicating a good condition of the used lymphocytes. For each experimental group of mice the number of spots/ $1 \times 10^6$  cells are illustrated in Figure 1A and 1B.

While the injection of OVA<sub>257-264</sub>-peptide with poly-L-arginine or CpG-ODN alone leads to low numbers of peptide-specific IFN- $\gamma$ -producing cells, the injection of OVA<sub>257-264</sub>-peptide with the combination of poly-L-arginine and CpG-ODN strongly enhances the peptide-specific response. Using the non-immunogenic Non-CpG-ODN instead of CpG-ODN, the co-application of poly-L-arginine has no increasing effect on the peptide-specific immune response which is rather low.

**Example 2 :** The combined application of CpG-ODN and poly-L-arginine strongly enhances the induction of T cells specific for a mastocytoma-derived peptide, a circum-sporozoite-derived peptide, a listeriolysin-derived peptide and a MHC class II restricted ovalbumin-derived peptide.

Mice	DBA/2 (Harlan/Olac)
Peptides	<p>a. Mouse mastocytoma P815-derived peptide P1A (LPYLGWLVF), restricted to MHC class I (H2-Ld) (Lethe et al., 1992).</p> <p>b. CSP-peptide (SYVPSAEQI) derived from the circumsporozoite protein of plasmodium yoelii (Rodrigues et al., 1992), restricted to MHC class I (H2-Kd).</p> <p>c. LLO-peptide (GYKDGNEYI) derived from listeriolysin O 91-99 of <i>Listeria monocytogenes</i> (Pamer et al., 1991), restricted to MHC class I (H2-Kd).</p> <p>d. OVA<sub>323-336</sub>-Peptide (ISQAVHAAHAEINE) derived from</p>

chicken ovalbumin, restricted to MHC class II (I-Ad) (Shimonkevitz et al., 1984).

All peptides were synthesized by standard solid phase F-moc synthesis, HPLC purified and analysed by mass spectroscopy for purity.

Dose: 300 µg/mouse

Poly-L-Arginine 60 (pR60)

Poly-L-Arginine with an average degree of polymerization of 60 arginine residues; SIGMA chemicals  
Dose: 100 µg/mouse

CpG-ODN 1668

thiophosphate substituted ODNs containing a CpG motif: TCC ATG ACG TTC CTG ATG CT, synthesized by NAPS GmbH, Göttingen.  
Dose: 5 nmol/mouse

Non-CpG-ODN 1911

thiophosphate substituted ODNs containing no CpG motif: TCC AGG ACT TTC CTC AGG TT, synthesized by NAPS Göttingen GmbH.  
Dose: 5 nmol/mouse

Experimental groups (5 mice per group)

1. PlA-Peptide + CpG-ODN + pR 60 100 µg
2. PlA-Peptide + Non-CpG-ODN + pR 60 100 µg
3. PlA-Peptide + CpG-ODN
4. PlA-Peptide + pR 60 100 µg
  
5. CSP-Peptide + CpG-ODN + pR 60 100 µg
6. CSP-Peptide + Non-CpG-ODN + pR 60 100 µg
7. CSP-Peptide + CpG-ODN
8. CSP-Peptide + pR 60 100 µg
  
9. LLO-Peptide + CpG-ODN + pR 60 100 µg
10. LLO-Peptide + Non-CpG-ODN + pR 60 100 µg
11. LLO-Peptide + CpG-ODN
12. LLO-Peptide + pR 60 100 µg
  
13. OVA-Peptide + CpG-ODN + pR 60 100 µg
14. OVA-Peptide + Non-CpG-ODN + pR 60 100 µg
15. OVA-Peptide + CpG-ODN

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## 16. OVA-Peptide + pR 60 100 µg

On day 0 mice were injected into each hind footpad with a total volume of 100 µl, 50 µl per footpad, containing the above mentioned compounds. Animals were sacrificed 4 days after injection and popliteal lymph nodes were harvested. Lymph nodes and (Shimonkevitz et al., 1984) were prepared as described in example 1 and IFN-γ-ELISPOTs were prepared. For each experimental group of mice the number of spots/1x10<sup>6</sup> cells are illustrated in Figure 2A-D.

While the injection of the peptides with poly-L-arginine or CpG-ODN alone leads to no or only low numbers of peptide-specific IFN-γ-producing cells, the injection of peptides with the combination of poly-L-arginine and CpG-ODN induces (e.g. CSP) or strongly enhances the peptide-specific response. Using the non-immunogenic Non-CpG-ODN instead of CpG-ODN, the co-application of poly-L-arginine has no increasing effect on the peptide-specific immune response which is rather low.

**Example 3 :** The combined application of CpG-ODN and poly-L-arginine (pR 60) synergistically enhances the immune response against MC1R (melanocyte stimulating hormone receptor).

Mice	C57Bl/6 (Harlan/Olac)
Peptide	MC1R-peptide (WGPF <del>F</del> LHL, F. Mattner, not published), a MHC class I (H-2Kb)-restricted epitope of melanocyte stimulating hormone receptor (MC1R) synthesized by standard solid phase F-moc synthesis, HPLC purified and analysed by mass spectrometry for purity. Dose: 300 µg/mouse
Poly-L-Arginine 60 (pR60)	Poly-L-Arginine with an average degree of polymerization of 60 arginine residues; SIGMA chemicals Dose: 1000, 100 or 10 µg/mouse
CpG-ODN 1668	thiophosphate substituted containing a CpG motif: TCC ATG <u>ACG</u> TTC CTG ATG CT, synthesized by NAPS Göttingen GmbH.

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Dose: 5 nmol/mouse

Experimental groups (3 mice per group)

1. MC1R + CpG-ODN + pR
2. MC1R + CpG-ODN
3. MC1R + pR

At day 0 mice were injected into each hind footpad with a total volume of 100  $\mu$ l (50  $\mu$ l per footpad) containing the above mentioned compounds. Animals were sacrificed 4 days after injection and popliteal lymph nodes were harvested. Lymph nodes and IFN- $\gamma$ -ELISPOTs were prepared as described in example 1. For each experimental group of mice the number of spots/ $1 \times 10^6$  cells are illustrated in Figure 3, standard deviation of ex vivo-stimulated triplicates were given.

While the injection of MC1R-peptide with poly-L-arginine or CpG-ODN alone leads to low numbers of peptide-specific IFN- $\gamma$ -producing cells, the injection of MC1R-peptide with the combination of poly-L-arginine and CpG-ODN strongly enhances the peptide-specific response.

**Example 4 :** The combined application of CpG-ODN and poly-L-arginine (pR 60) induces at different injection sites a strong antigen specific immune response against Ovalbumin-peptide.

Mice	C57Bl/6 (Harlan/Olac)
Peptide	OVA <sub>257-264</sub> -Peptide (SIINFEKL), a MHC class I (H-2Kb)-restricted epitope of chicken Ovalbumin (Rotzschke et al., 1991), synthesized by standard solid phase F-moc synthesis, HPLC purified and analysed by mass spectroscopy for purity. Dose: 300 $\mu$ g/mouse
Poly-L-Arginine 60 (pR60)	Poly-L-Arginine with an average degree of polymerization of 60 arginine residues; SIGMA chemicals Dose: 100 $\mu$ g/mouse
CpG-ODN 1668	thiophosphate substituted ODNs containing a CpG motif: TCC ATG <u>ACG</u> TTC CTG ATG CT, synthesized

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by NAPS Göttingen GmbH.

Dose: 5 nmol/mouse

Experimental groups (5 mice per group) injection sites:

1. footpad
2. s.c. /flank
3. intra pinna

At day 0 mice were injected (Ovalbumin-peptide + CpG-ODN + pR 60) into the different injection sites with a total volume of 100  $\mu$ l (50  $\mu$ l per footpad) containing the above mentioned compounds. Animals were sacrificed 4 days after injection and the draining lymph nodes were harvested. Lymph nodes and IFN- $\gamma$ - ELISPOTs were prepared in triplicates (standard deviation is given) as described in example 1. For each experimental group of mice the number of spots/ $1 \times 10^6$  cells are illustrated in Figure 4.

The co-application of OVA<sub>257-264</sub>-peptide with poly-L-arginine and CpG-ODN induces at different injection sites a strong antigen specific immune response. The intra pinna injection is superior to the other subcutaneous injections (footpad, flank).

**Table 2****Peptide antigens used for vaccination experiments**

<i>peptide</i>	<i>sequence</i>	<i>source</i>	<i>restricted to</i>	<i>publication</i>
OVA <sub>257-264</sub>	SIINFEKL	Chicken Ovalbumin	MHC class I, H2Kb	(Rotzschke et al., 1991)
P1A	LPYLGWLVF	Mouse mastocytoma P815	MHC class I, H-2Kd	(Lethe et al., 1992)
CSP	SYVPSAEQI	Circumsporozoite protein Plasmodium yoelii	MHC class I, H-2Kd	(Rodrigues et al., 1992)
LLO	GYKDGNEYI	Listeriolysin Listeria monocytogenes	MHC class I, H2kd	(Pamer et al., 1991)
OVA <sub>323-336</sub>	ISQAVHAA-HAEINE	Chicken Ovalbumin	MHC class II, I-Ad	(Shimonkevitz et al., 1984)
MC1R	WGPFFLHL	melanocyte stimulating hormone receptor	MHC class I, H-2Kb	F. Mattner, not published

**Example 5 :** The combined application of CpG-ODNs and poly-L-arginine (pR 60) prevents the induction of systemic TNF- $\alpha$  and IL-6-production.

**Mice** C57B1/6 (Harlan/Olac)

**Peptide** OVA<sub>257-264</sub>-peptide (SIINFEKL, a MHC class I (H-2Kb)-restricted epitope of chicken ovalbumin (Rotzschke et al., 1991), was synthesized using standard solid phase F-moc synthesis, HPLC-purified and analysed by mass spectroscopy for purity  
Dose: 300  $\mu$ g/mouse

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**Poly-L-Argini-**

ne 60 (pR60) Poly-L-arginine with an average degree of polymerization of 60 arginine residues; SIGMA chemicals

Dose: 100 µg/mouse

CpG-ODN 1668 thiophosphate substituted ODNs containing a CpG motif: TCC ATG ACG TTC CTG ATG CT, synthesized by NAPS GmbH, Göttingen.

Dose: 5 nmol/mouse

**Experimental groups** (4 mice per group)

1. OVA<sub>257-264</sub>-peptide
2. pR60
3. CpG 1668 + OVA<sub>257-264</sub>-peptide
4. CpG 1668 + pR60 + OVA<sub>257-264</sub>-peptide

Mice were injected into each hind footpad with a total volume of 100 µl, 50 µl per footpad, containing the above mentioned compounds. One hour after injection blood was taken via the tail-vein and serum was prepared. The amount of proinflammatory cytokines (TNF-α and IL-6) in the sera was determined using cytokine-specific ELISAs.

**TNF-α/IL-6 in sera / 1 h after injection****injection 245+**

	<b>TNF-α (pg/ml)</b>	<b>IL-6 (pg/ml)</b>
-	0	0
+pR (100 µg)	0	0
CpG 1668 (5nMol)	171,5	33,1
CpG 1668 (5nMol)+pR (100µg)	0	0



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## C l a i m s :

1. Pharmaceutical composition comprising
  - an antigen,
  - an immunogenic oligodeoxynucleotide containing CpG motifs (CpG-ODN), and
  - a polycationic polymer.
2. Composition according to claim 1, characterized in that the antigen is a protein derived from a viral, parasitic or bacterial pathogen.
3. Composition according to claim 1, characterized in that the antigen is a tumor antigen.
4. Composition according to claim 1, characterized in that the antigen is an autoimmune antigen.
5. Composition according to any one of claims 1 to 4, characterized in that the polycationic compound is a basic polypeptide, an organic polycation, a basic polyaminoacid or mixtures thereof.
6. Composition according to any one of claims 1 to 5, characterized in that the polycationic compound is polylysine, polyarginine, a polypeptide containing more than 50 % of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof.
7. Composition according to any one of claims 1 to 6, characterized in that the polycationic compound is derived from the REV-protein or the TAT-protein of HIV, chitosan or other chitin derivatives.
8. Composition according to any one of claims 1 to 7, characterized in that the immunogenic CpG-ODN is selected from synthetically produced DNA molecules, thiophosphate substituted ODNs, immunogenic insect DNA, recombinant DNA molecules, DNA molecules containing a CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines, or mixtures thereof.

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9. Vaccine, comprising a composition according to any one of claims 1 to 8.

10. Use of a composition according to any one of claims 1 to 8 for manufacturing a vaccine.

11. Kit comprising

- a component containing an immunogenic CpG-ODN, and
- a component containing a polycationic polymer and
- a component containing an antigen.

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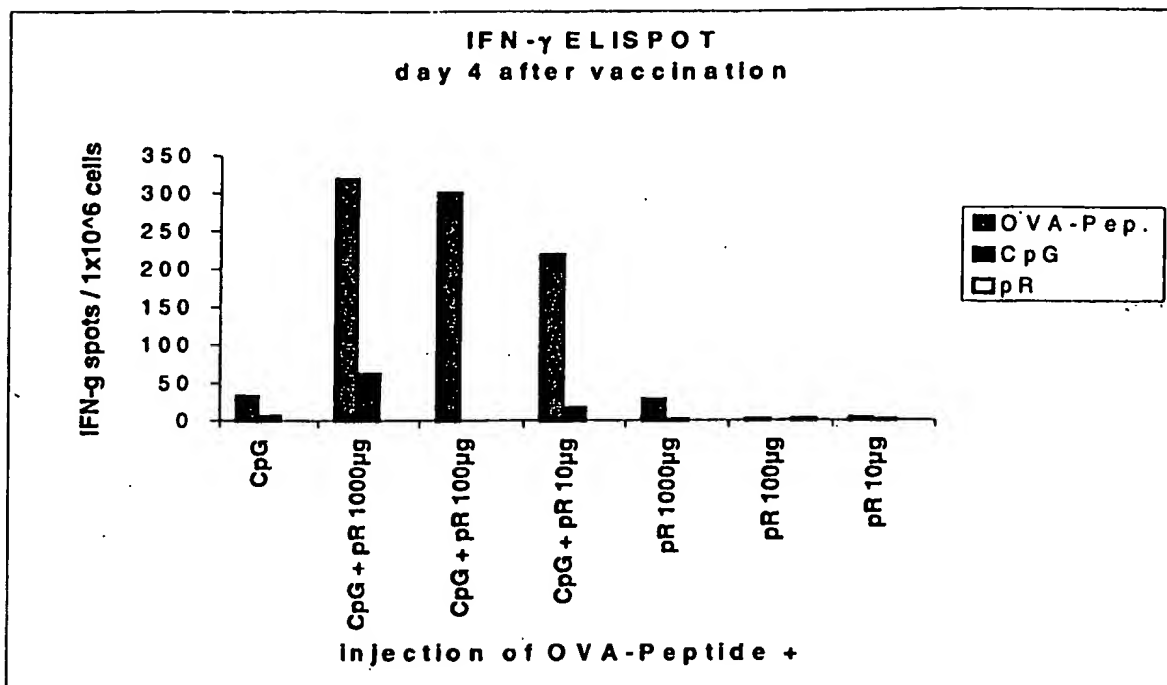


Fig. 1A

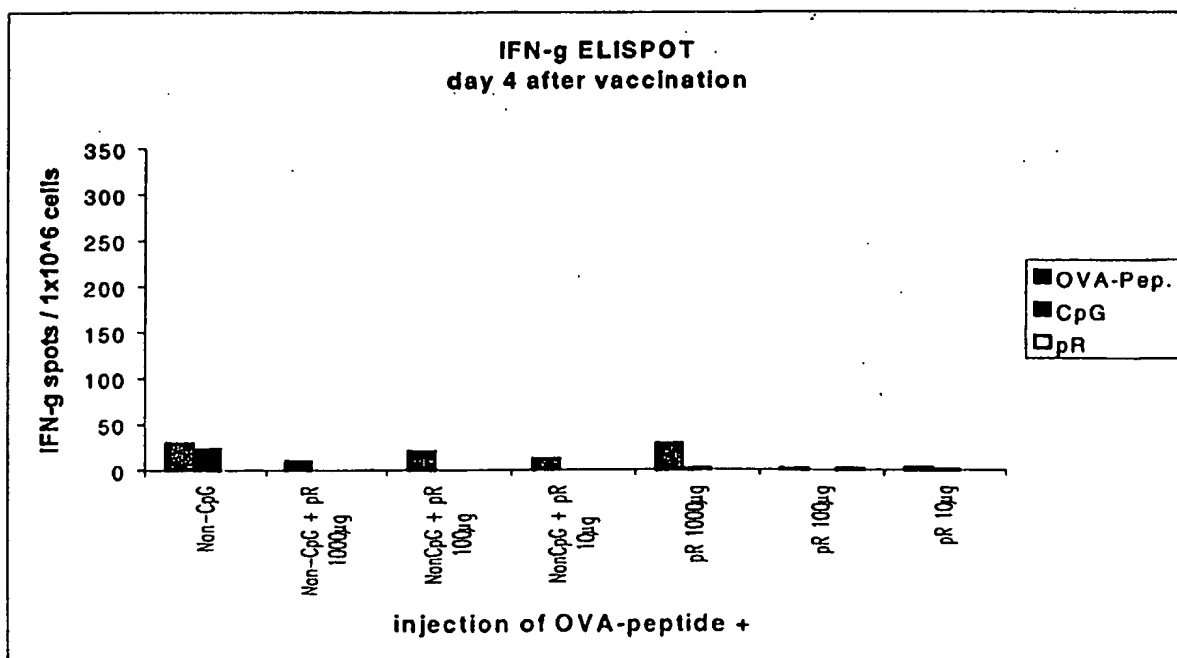


Fig. 1B



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Fig. 2A

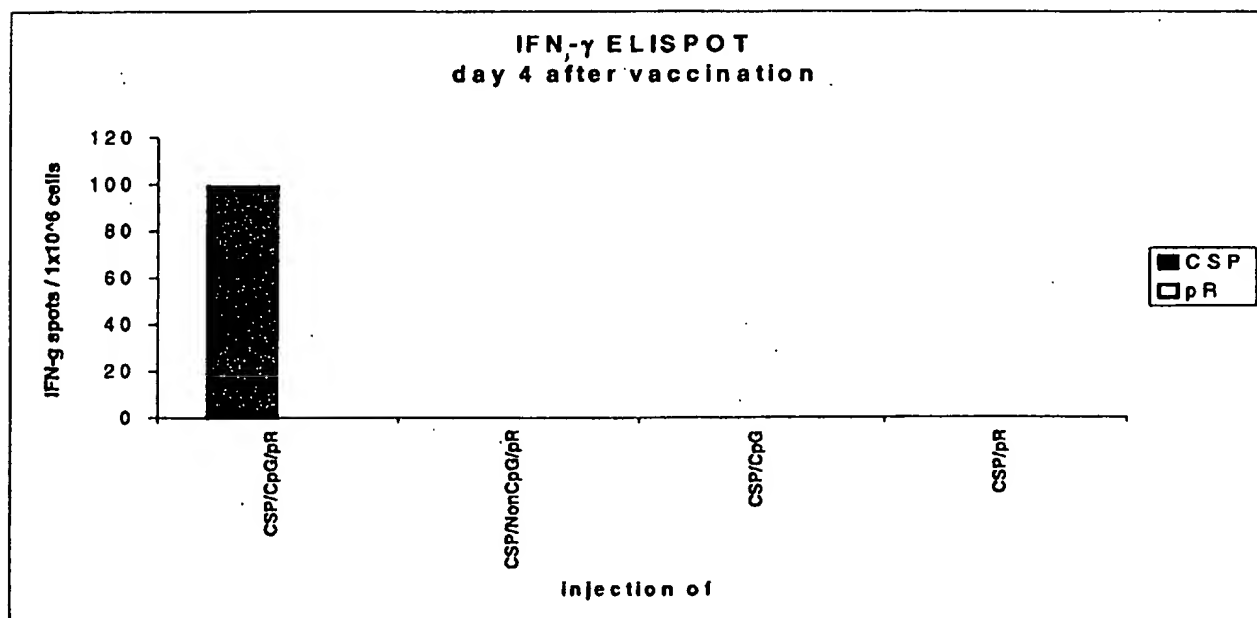
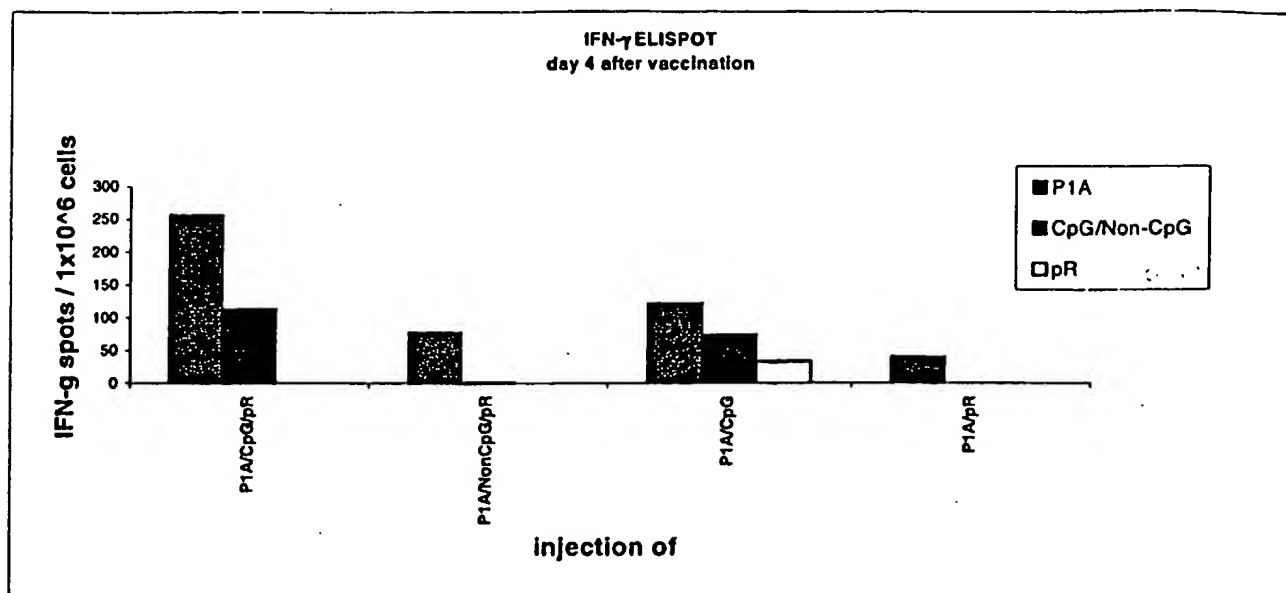


Fig. 2 B

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Fig. 2C

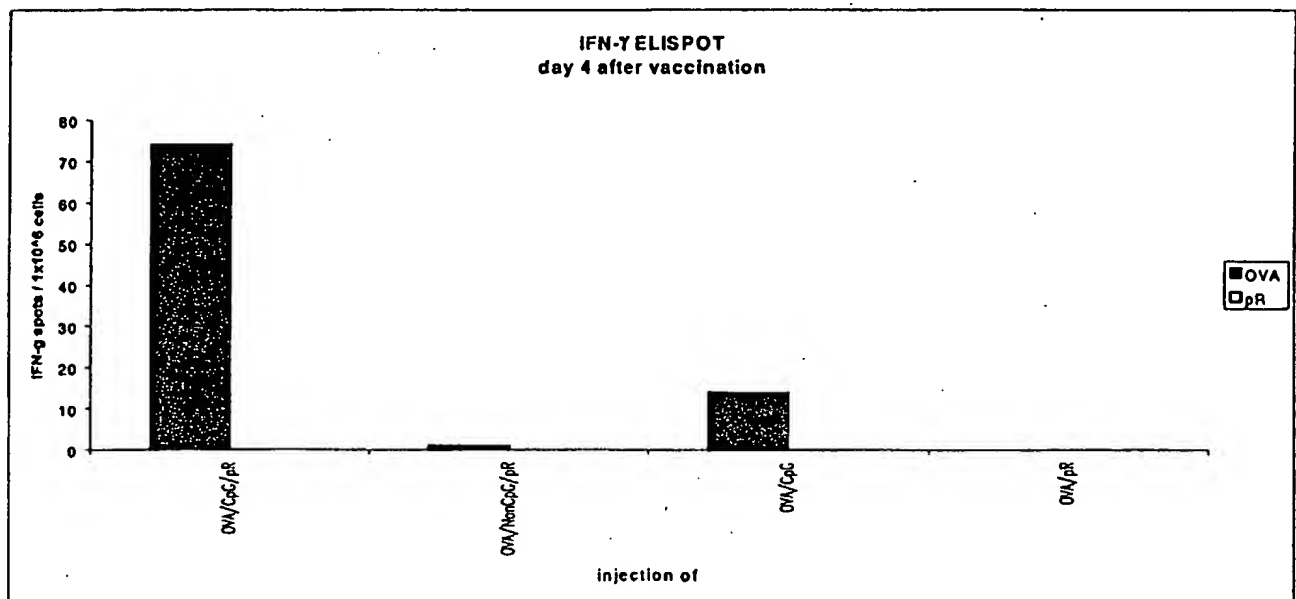
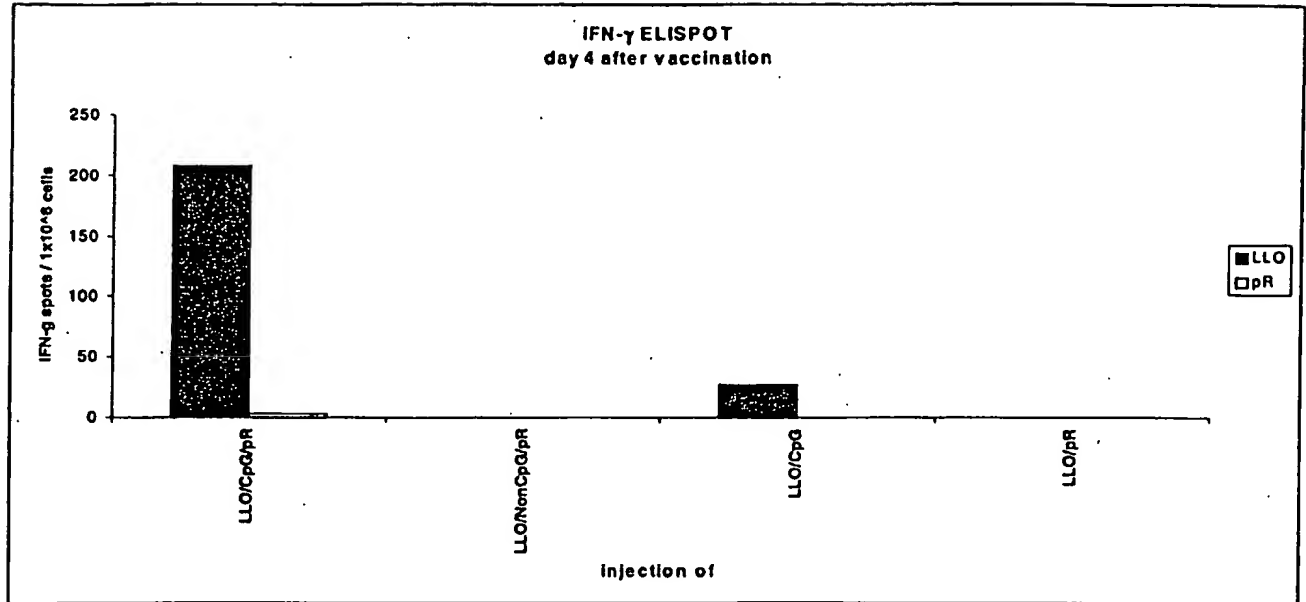


Fig. 2D

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Fig. 3

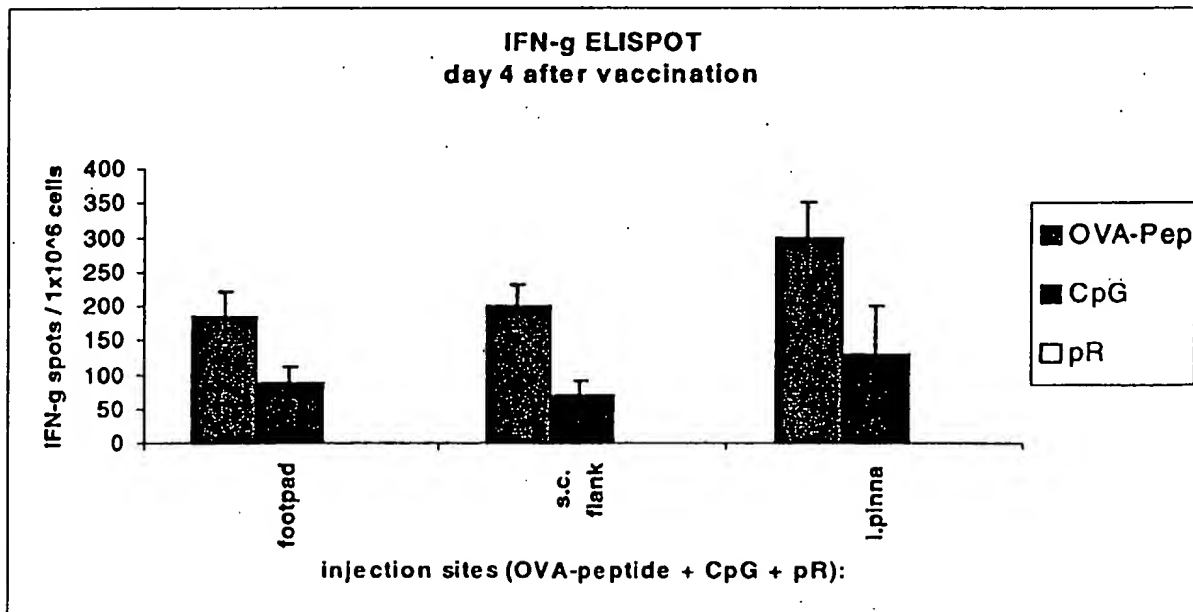
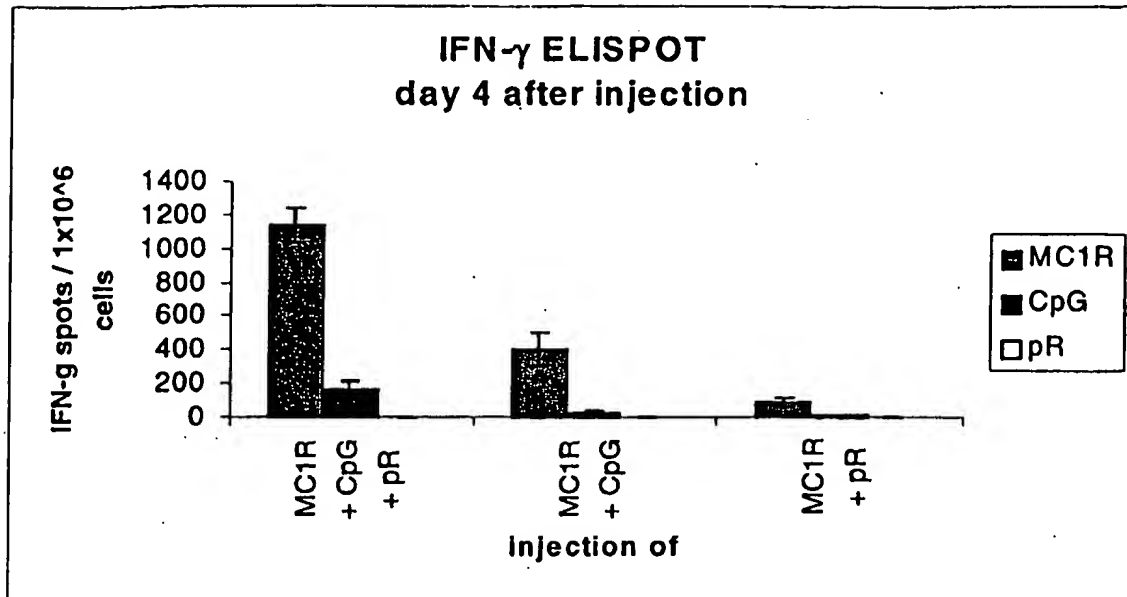


Fig. 4

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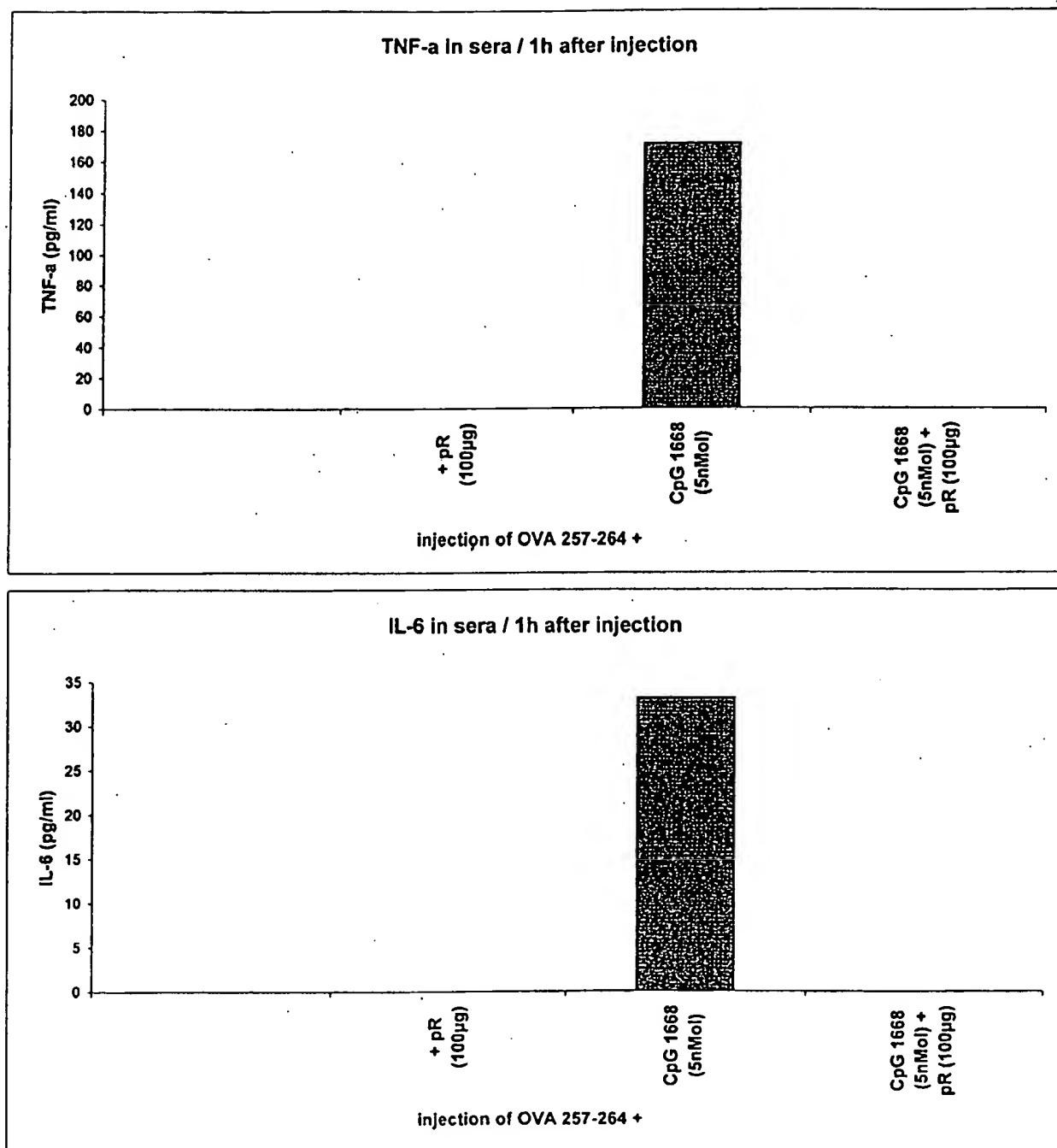


Fig.5

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/39 A61K39/002 A61K39/02 A61K39/12 A61P35/00  
 A61P37/00 A61K31/722 A61K31/7125 C07K14/34 //C07K14/155

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, EMBASE, CHEM ABS Data, LIFESCIENCES, PAJ, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 38528 A (BIRNSTIEL MAX ;BUSCHLE MICHAEL (AT); SCHMIDT WALTER (AT); BOEHRING) 5 August 1999 (1999-08-05) page 8; claim 1 ---	1-11
A	WO 97 30721 A (SCHWEIGHOFFER TAMAS ;BIRNSTIEL MAX (AT); BUSCHLE MICHAEL (AT); SCH) 28 August 1997 (1997-08-28) abstract --- -/--	1-11



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Date of the actual completion of the international search

19 June 2001

Date of mailing of the international search report

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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